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The Left-Right Coordinator: The Role of Vg1 in Organizing Left-Right Axis Formation

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Summary

The asymmetries of internal organs are consistently oriented along the left-right axis in all vertebrates, and perturbations of left-right orientation lead to significant congenital disease. We propose a model in which a "left-right coordinator" interacts with the Spemann organizer to coordinate the evolutionarily conserved three-dimensional asymmetries in the embryo. The *Vg1* cell-signaling pathway plays a central role in left-right coordinator function. Antagonists of Vg1 alter left-right development; antagonists of other members of the TGFβ family do not. Cell-lineage directed expression of Vg1 protein can fully invert the left-right axis (situs inversus), can randomize left-right asymmetries, or can "rescue" a perturbed left-right axis in conjoined twins to normal orientation (situs solitus), indicating that Vg1 can mimic left-right coordinator activity. These are the first molecular manipulations in any vertebrate by which the left-right axis can be reliably controlled.

Introduction

The development of the left-right axis is a highly conserved process in vertebrates by which the orientation of heart looping and gut coiling are coordinated with the anterior-posterior and dorsal-ventral axes. Disruptions of left-right axis formation are associated with cardiac and visceral defects and are responsible for significant mortality and morbidity in human infants (Bowers et al., 1996). Recent advances have identified genes involved in the left-right pathway, but not the mechanisms that initiate the left-right axis.

Although left-right asymmetry is not revealed morphologically until relatively late in embryonic development, a number of genes are expressed asymmetrically along the left-right body axis prior to left-right morphogenesis (Levin, 1997). One of these genes, *nodal* (a TGFβ family member), is relatively conserved in its asymmetric expression patterns across species (Levin et al., 1995; Collignon et al., 1996; Hyatt et al., 1996; Lowe et al., 1996; Lustig et al., 1996). Prior to cardiac looping, *nodal* is expressed in left lateral plate mesoderm near the cardiac primordia, but not in the right lateral plate mesoderm, suggesting that *nodal* could be responsible for signaling spatial cues to the developing heart.

Several experimental and genetic manipulations implicate the embryonic midline in left-right development. Mice homozygous for mutations in the *IV* gene, which encodes an axonemal dynein heavy chain that is expressed in the midline (Supp et al., 1997) have randomized cardiac and visceral orientation ("heterotaxia") and have a variable pattern of *nodal* expression (Lowe et al., 1996). Genetic mutations in zebrafish that disrupt the formation of the dorsal midline randomize left-right cardiac orientation (Danos and Yost, 1996; Chen et al., 1997). In *Xenopus* embryos, extirpation of midline cells results in randomized left-right cardiac orientation and bilateral expression of *nodal* (Danos and Yost, 1996; Lohr et al., 1997). These results indicate that midline cells, which are derived from the Spemann organizer, link left-right development with development along the other embryonic axes (Danos and Yost, 1995).

Asymmetric ectopic expression of several genes can both alter *nodal* expression patterns and randomize left-right development. In 16-cell *Xenopus* blastula, injection of a chimeric RNA that expresses mature Vg1 protein in the right dorsovegetal cells randomizes cardiac and gut orientation and induces bilateral *nodal* expression (Hyatt et al., 1996). During the gastrula stages in chick embryos, ectopic gene expression of *activin* or *shh* on the side contralateral to their normal expression (Levin et al., 1995, 1997) or reduction of chick *snail-related* gene (cSnR) (Isaac et al., 1997) randomizes cardiac left-right orientation. Direct misexpression of *nodal* in *Xenopus* gastrula randomizes cardiac orientation (Sampath et al., 1997). Ectopic expression of *nodal* in chick, though capable of randomizing cardiac orientation, can also result in bilaterally symmetric hearts (Levin et al., 1997). In cases of bilateral symmetry in the left-right pathway, the organ primordia are usually capable of generating morphological asymmetry (e.g., the cardiac tube still loops); however, the orientation of the asymmetric organs with respect to the other body axes is lost.

In contrast to several experimental and genetic manipulations that randomize left-right orientation, full inversion of the left-right axis is seen only in humans with situs inversus totalis and in *inv/inv* homozygous mouse embryos (Yokoyama et al., 1993). Approximately 85% of *inv/inv* embryos are mirror-images of normal embryos; the internal organs are in the opposite orientation from normal, and *nodal* is expressed on the right side and not on the left side (Collignon et al., 1996; Lowe et al., 1996). This reversal rate is statistically equivalent to full inversion of the left-right axis. The *inv* mutation is a result of a transgene insertion and complex chromosome rearrangements, and the *inv* gene has not been molecularly identified (Yokoyama et al., 1993). It has been previously assumed that inversion of the left-right axis would require a two-step process, both eliminating a signaling pathway on one side and ectopically activating that pathway on the contralateral side. Models of *inv/inv* include proposals that a default pathway directs

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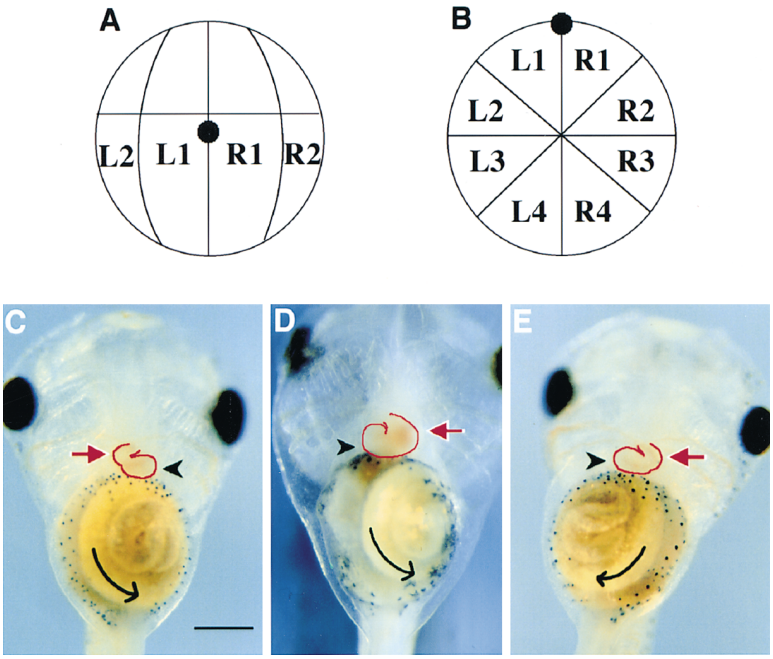

Figure 1. Cell Lineage Targets and Left-Right Phenotypes

Cell designations of 16-cell *Xenopus* embryos. L, left; R, right; numbers 1 through 4 indicate dorsal-most to ventral-most cells. The black dot represents the Nile blue dot used to mark the dorsal midline, which when bisected by a cleavage plane divides the embryo into equal left and right halves. Black bar represents 0.5 mm in all figures.

(A) Cell designations, dorsal view.

(B) Cell designations, vegetal view. This is the view in which data is presented in subsequent figures.

(C) Situs solitus. Ventral view of stage 46 embryo with normal heart orientation (outlined in red), with outflow tract (red arrow) to the embryo's right and ventricle (black arrowhead) to the left, and normal gut orientation with counterclockwise coiling (black arrow). Embryo was injected in L3 with *pXEX-BVg1*.

(D) Heterotaxia. Ventral view of stage 46 embryo with reversed heart orientation (outlined in red), the outflow tract (red arrow) looped to the embryo's left side and the ventricle (black arrowhead) to the right, and a normally coiled gut. The direction of gut coiling is statistically randomized in embryos with reversed left-right cardiac orientation. Embryo was injected in R3 with *activin* RNA. The phenotype of independent orientation of heart and gut was also seen in *BVg1* injections into R1 (Hyatt et al., 1996).

(E) Situs inversus totalis. Ventral view of stage 46 embryo with reversed heart orientation and reversed gut orientation (clockwise coiling). Embryo was injected in R3 with *pXEX-BVg1*.

the left-right axis in the opposite orientation (Yokoyama et al., 1993) or that asymmetric sister chromatid segregation plays a role in left-right axis formation (Klar, 1994). Based on present results, we propose a more parsimonious model for the *inv* mutation.

Molecular manipulations described in the present study reach a never-before achieved goal, the predictable generation of embryos in any of three categories of left-right development: normal orientation, randomization, or inverted orientation. Expression of Vg1 in a specific lineage on the right side can invert the left-right axis, simultaneously giving the geometric right side a "left identity" and the geometric left side a "right identity." Expression of Vg1 on the left side of an otherwise randomized embryo "rescues" the left-right axis to normal, and expression of Vg1 on the right side of an otherwise randomized embryo inverts the left-right axis. In addition, results with other members of the TGFβ family, with a dominant negative receptor for TGFβs, and with TGFβ family signaling antagonists serve to eliminate the possibility that Vg1 is mimicking signals from other known TGFβ family members. Based on these findings, we propose that a "Left-Right coordinator" is positioned early in development on the prospective left side of the embryo and, via *Vg1* signaling, is capable of establishing both left-side and right-side identities.

Results

Inversion of the Left-Right Axis

Vg1 is a member of the TGFβ family of cell-signaling factors and has been implicated both in mesoderm formation along the dorsal-ventral axis (Dale et al., 1993; Thomsen and Melton, 1993) and in left-right development (Hyatt et al., 1996). Like other members of the TGFβ family, Vg1 is synthesized as a proprotein that appears to be processed to an active mature form in a restricted region of the *Xenopus* embryo (Dale et al., 1993; Thomsen and Melton, 1993). During oogenesis, *Vg1* RNA is synthesized and localized to the vegetal hemisphere so that it is inherited in vegetal cells in blastula-stage embryos. Vg1 proprotein is readily detected in left and right cells of blastula-stage embryos (data not shown), but mature protein is difficult to detect in either normal embryos or in embryos injected with *Vg1* RNA (Dale et al., 1993; Thomsen and Melton, 1993). Mature Vg1 protein can be made in detectable amounts by injection of chimeric RNAs that provide different processing sites, derived from other members of the TGFβ family, fused to the coding region of the mature Vg1 peptide (Dale et al., 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995). For example, BVg1 is a chimera that provides mature Vg1 peptide via the BMP protein processing pathway and AVg is a chimera utilizing the activin pathway.

To determine whether *Vg1* is involved in left-right axis formation, *BVg1* RNA was injected into distinct vegetal cell lineages of the 16-cell *Xenopus* embryo. Cell nomenclature and an overview of left-right morphological phenotypes are given in Figure 1. *BVg1* had strikingly different effects depending on where it was expressed. Injection of *BVg1* RNA into R3, the right lateral vegetal cell, resulted in left-right reversal rates that are similar to those seen in *inv/inv* mice and that are statistically equivalent to full inversion of the left-right axis (Figure 2A). In contrast, *BVg1* injection into R1 randomizes the left-right orientation of cardiac looping and visceral coiling, analogous to heterotaxia in humans (Hyatt et al., 1996). Injection of *BVg1* RNA into any cell on the left side (L1, L2, or L3) had no significant effect on left-right

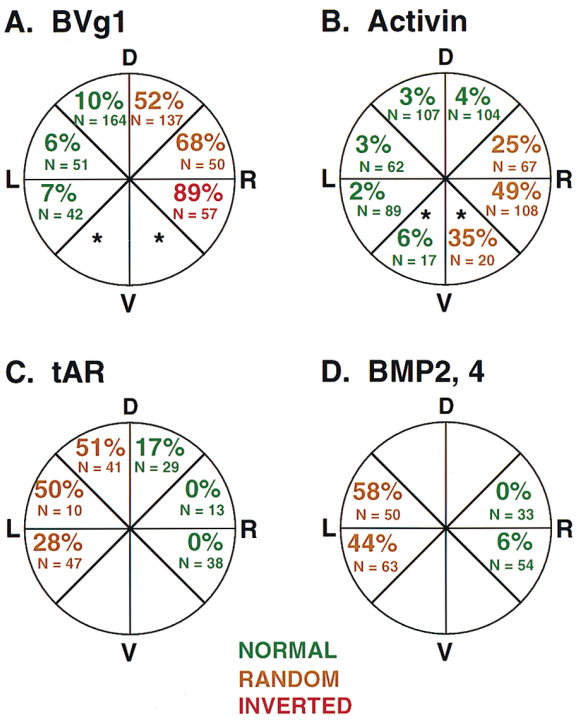

Figure 2. Cardiac Left-Right Reversal Rates from Lineage-Targeted Injections

The percentage of injected embryos that displayed cardiac reversal and the number of scored embryos (N) are indicated in vegetal-view diagrams of cell lineages (see Figure 1). Results in green indicate statistical equivalence ($p < 0.05$) to normal cardiac reversal rates, brown indicates statistical equivalence to randomization, and red indicates statistical equivalence to full inversion of left-right orientation. Asterisk indicate injections in ventral cells that result in twinning. Blank cells indicate not determined. Uninjected embryos or embryos injected with control RNAs have a cardiac reversal rate of up to 8% depending on the batch of eggs (Hyatt et al., 1996; Lohr et al., 1997).

(A) *BVg1* injection. Similar to *BVg1*, injection of *AVg* into R3 inverted the left-right axis. Control RNA injections of either *Vg1* RNA or *BVg1–2cx* (a form of *BVg1* that can not be proteolytically processed into mature Vg1) into L3 and R3 had no effect on left-right development ($N > 23$ for each). These results confirm the hypothesis that Vg1 expression along the left-right axis is regulated at the level of proprotein processing (Hyatt et al., 1996).

(B) Activin injection. Heart and gut orientation was randomized by injection in R2, R3, and R4.

(C) Dominant negative receptor *tAR* injection. Heart and gut orientation was randomized in left-side injections. Results from L2 and L3 are not statistically different ($p < .05$). Right-side injections did not perturb organ orientation or *Xnr-1* expression pattern. Data from R1 and L1 (Hyatt et al., 1996) are shown for comparison.

(D) *BMP4* injection. Of embryos with heart reversals, only 4/15 had gut reversals. Results of *BMP2* injections were similar to *BMP4*; L2 (61%, N = 28) and R2 (4%, N = 46). Injection of *BMP2* or *BMP4* into the L1 and R1 cells had other effects on development, most likely owing to their strong antagonism of the Spemann organizer (Dale et al., 1992; Sasai et al., 1995).

development (Figure 2A). Expression of *BVg1* shortly after the mid-blastula transition, by injection of *pXEX-BVg1*, gave similar results. For example, 75% of embryos injected in R3 with *pXEX-BVg1* had reversed heart orientation (N = 116); of those with reversed hearts, 90% had reversed gut orientation, indicating that the entire left-right axis was inverted (Figure 1E). Injections of *pXEX-BVg1* into L3 (N = 109) or control vector *pXEX* into either L3 (N = 24) or R3 (N = 23) had no significant effect on left-right development (Figure 1C). These results suggest that the *Vg1* signaling pathway is competent to establish the left-right axis at least throughout the blastula stages. The ability of Vg1 to invert the left-right axis when expressed in the R3 cell and to randomize when expressed in the R1 cell indicates that the specific lineage or location of Vg1 expression determines the left-right axis. The ability to invert the left-right axis by expression of Vg1 protein in R3 suggests that the highest levels of endogenous mature Vg1 expression are in L3.

Expression of *Activin* RNA in a Subset of Right Cells Induces Heterotaxia but Not Left-Right Inversion

It was not known whether the effects of *BVg1* injection are specific to Vg1 or can be mimicked by other members of the TGFβ family. Activin is closely related to Vg1 and has been implicated in the regulation of cell identity in *Xenopus* (Fukui et al., 1993; Slack, 1994). In contrast to the left-right axis inversions caused by injection of *BVg1*, injection of *activin* RNA into the R3 cell only randomized the direction of cardiac looping (Figure 2B). Injection of *activin* RNA into either the R2 or R4 cell also altered the orientation of cardiac looping, producing a reversal rate lower than randomization but significantly higher than background reversal rates (Figure 2B). Injections into other cells (L1–L4 and R1) had no significant effect on left-right development (Figure 2B).

It is striking that ectopic expression of *activin* in chick embryos (Levin et al., 1995) or in the R3 cell in *Xenopus* (Figure 2B) randomizes left-right development, whereas *activin* RNA injection in the R1 cell does not (Hyatt et al., 1996). One possible explanation for why *activin* RNA injections into the R1 cell do not randomize left-right development is that activin protein is not processed in the R1 cell. To assess the activin processing pathway functions in R1, we asked whether active Vg1 protein could be produced via the activin processing pathway. *AVg* is a chimeric construct that contains the activin predomain and activin processing site fused to the Vg1 mature domain (Kessler and Melton, 1995). Injection of *AVg* RNA into the R1 cell reversed the orientation of cardiac looping in 37% of the cases (N = 19), while injection of *AVg* RNA into the L1 cell had little effect on left-right development (11%, N = 18). Thus, similar to *BVg1* injections, *AVg* injections into R1 cells altered left-right development. This indicates that the activin protein processing pathway is functional in R1 cells and that the left-right effects of Vg1 expression in R1 are specific to the action of Vg1 protein, regardless of whether it is generated via the BMP2 or the activin processing pathway.

In addition to randomizing the direction of cardiac looping, *activin* RNA injection also randomized the left-right orientation of the viscera. In embryos in which cardiac orientation was reversed by injection of *activin*, the orientation of gut coiling was randomized (Figure 1D). Embryos with reversed hearts due to *activin* injection into R2, R3, or R4 had reversed gut orientation in 43% (N = 7), 63% (N = 24), and 67% (N = 6), respectively.

These results are statistically equivalent to independent assortment (discordance) of heart and gut orientation, indicating that with ectopic activin expression individual organs independently align their left-right orientation, similar to heterotaxia syndromes seen in humans (Bowers et al., 1996).

Altered *Xnr-1* Expression Is Correlated with Cardiac Orientation

Nodal gene asymmetric expression in left lateral plate mesoderm is conserved in vertebrates and serves as a useful marker for the left-right signaling pathway that precedes the specification of cardiac left-right orientation (Levin et al., 1995; Collignon et al., 1996; Hyatt et al., 1996; Lowe et al., 1996; Lustig et al., 1996). Perturbation of cardiac orientation by ectopic expression of *Xnr-1* in *Xenopus* suggests that this gene is upstream of cardiac orientation (Sampath et al., 1997). Therefore, it is predicated that injections that invert the left-right axis should invert *Xnr-1* expression patterns (i.e., *Xnr-1* expressed on the right side only), injections that give normal heart orientation should give normal expression of *Xnr-1* (left side only), and injections that randomize cardiac orientation should give intermediate patterns of *Xnr-1* expression.

In each of the three possible categories of left-right phenotypes (normal, randomized, and inverted), cardiac orientation is correlated with *Xnr-1* expression patterns. *Xnr-1* expression on the left is associated with normal cardiac orientation (Figures 3A, 3B, and 3E) and on the right with inverted cardiac orientation (Figures 3C and 3D). Randomized cardiac orientation is correlated with either *Xnr-1* expression on both sides (Figure 3F) or on neither side (as discussed below for *BMP2*, Figure 4). The correlation of *Xnr-1* expression patterns and cardiac orientation suggests that during the period of specification, the cardiac primordia are capable of interpreting quantitative differences in *Xnr-1* expression.

Disruption of Vg1 Signaling, but Not BMP or Activin Signaling, on the Left Side Randomizes Left-Right Development

Expression of Vg1 on the right side (R3) inverts the left-right axis, while expression of *Vg1* on the left side maintains the normal left-right axis (Figure 2A). If Vg1 normally regulates left-right axis formation, elimination of Vg1 signaling should give randomization of left-right asymmetries. It has been technically difficult to eliminate endogenous Vg1 from the *Xenopus* embryo, due to strong accumulation during oogenesis (B. A. H., J. Heasman, and H. J. Y., unpublished data). As an alternative approach, one can perturb Vg1 activity or the putative downstream signaling pathways that are utilized by Vg1. Four specific approaches eliminate other candidate molecules (activin and BMPs) and indicate that *Vg1* signaling is essential for left-right development: expression of a dominant negative receptor and expression of signaling antagonists noggin, follistatin, and BMP.

A truncated activin receptor (tAR), originally devised to eliminate signaling by activin, inhibits mesoderm induction by activin (Hemmati-Brivanlou and Melton, 1992), BVg1 (Schulte-Merker et al., 1994), and BMPs (Wilson and Hemmati-Brivanlou, 1995; Chang et al., 1997; Frisch and Wright, 1998) by interfering with ligand-induced receptor activation. Injection of *tAR* RNA into the left side, but not the right side, randomized the orientation of heart looping and gut coiling (Figure 2C). Correlated with its effects on organ orientation, injection of *tAR* RNA into the L1 cell altered *Xnr-1* in a complex fashion, resulting in embryos with either left-sided, bilateral, right-sided, or no *Xnr-1* expression in lateral plate mesoderm. This phenotype is analogous to the expression patterns of nodal in *iv/iv* mouse embryos in which left-right orientation is randomized (Collignon et al., 1996; Lowe et al., 1996). Disrupted left-right orientation by *tAR* expression indicates that endogenous signaling in left-side cells by *activin*, *BMPs*, or *Vg1* coordinates left-right axis formation.

Results with antagonists of activin and BMP eliminate these molecules as candidates for endogenous signaling on the left side. Noggin antagonizes BMP signaling

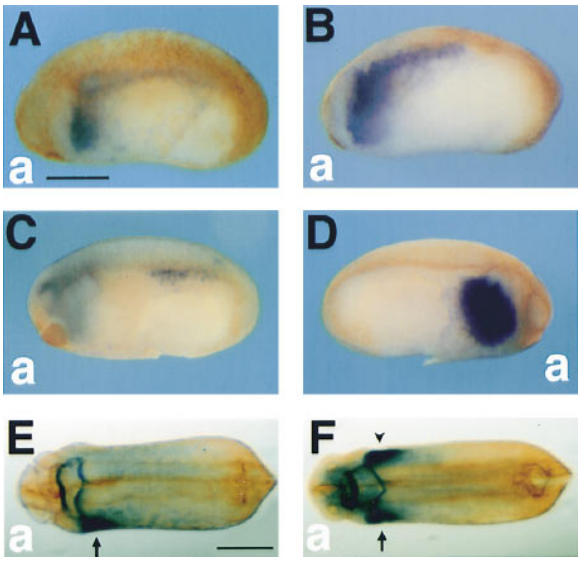

Figure 3. *Xnr-1* RNA Expression Patterns Are Correlated with Left-Right Orientation

Tailbud stage embryos were probed for *Xnr-1* by in situ hybridization. Anterior indicated by (a). (A–D) lateral view with dorsal at top; (E–F) dorsal view with right side at top.

(A) Uninjected embryo, left lateral view of normal *Xnr-1* expression pattern in left lateral plate mesoderm.

(B) *BVg1* injection into L3 results in normal left-right orientation and normal *Xnr-1* expression pattern.

(C) *Xnr-1* expression is absent in anterior left lateral plate mesoderm of embryos injected with *BVg1* in the R3 cell. Left view, same embryo as (D). The strong signal in the right lateral plate (see [D]) can be seen through the embryo. In concurrence with the cardiac inversion rates, 88% of the injected embryos (N = 44) displayed *Xnr-1* expression that was either exclusively in the right side or was significantly stronger in the right side than the left side.

(D) *Xnr-1* expression is induced in anterior right lateral plate mesoderm of embryos injected with *BVg1* in the R3 cell. Right view of embryo in (C).

(E) Normal left lateral plate expression of *Xnr-1* (arrow) in an embryo injected with activin in the L3 cell.

(F) Bilateral expression of *Xnr-1* in an embryo injected with *activin* RNA in R3. Bilateral *Xnr-1* expression is presumably due to endogenous expression on the left (arrow) and ectopic expression on the right (arrowhead).

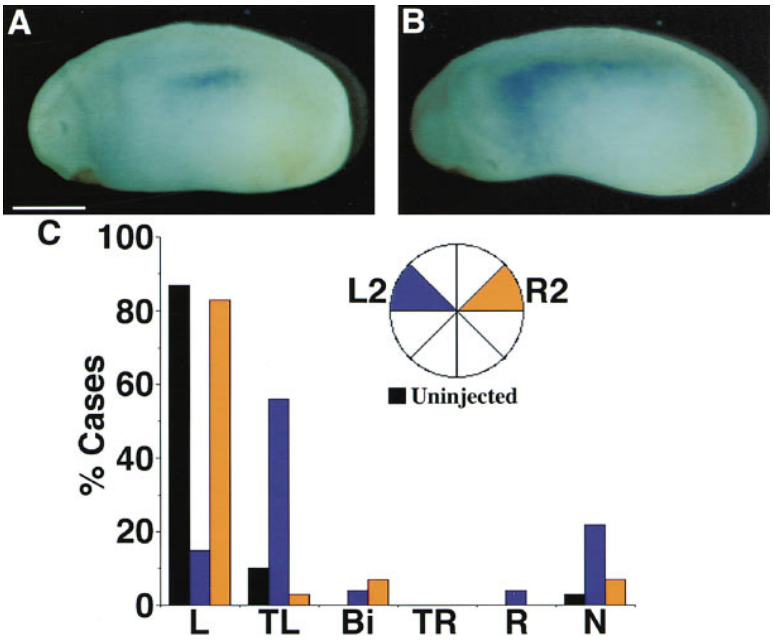

Figure 4. Injection of *BMP2* RNA into the L2 Cell Reduced the Anterior Domain of *Xnr-1* Expression and Randomized Cardiac Left-Right Orientation

(A) Embryo injected into the L2 cell with *BMP2* RNA truncated expression of *Xnr-1*. The anterior domain of *Xnr-1* expression is absent and the posterior domain is present. Lateral view, anterior to the left.

(B) Embryo injected into the R2 cell with *BMP2* RNA with normal expression of *Xnr-1*. The full expression domain of *Xnr-1* from anterior to posterior can be seen in this embryo.

(C) Graph of the percentage of cases of indicated *Xnr-1* expression in uninjected (black, N = 31), L2 injected (purple, N = 27), and R2 injected (orange, N = 29). Cases are defined as: L, left-only full expression; TL, truncated left-only expression; Bi, bilateral equal expression; TR, truncated right-only expression; R, right-only full expression; N, no expression.

in whole embryos and in cultured cell induction assays (Re'em-Kalma et al., 1995; Jones et al., 1996; Sasai et al., 1996; Wilson et al., 1997) by binding and inactivating BMPs (Zimmerman et al., 1996). Injection of *noggin* RNA does not significantly alter left-right development (Table 1A), indicating that endogenous *BMP* signaling is not necessary for early left-right development. Follistatin binds and antagonizes activin (Hemmati-Brivanlou et al., 1994) and BMP4 (Fainsod et al., 1997) in vitro and in vivo. Injection of *follistatin* RNA does not alter left-right development (Table 1A), indicating that both endogenous *BMP* signaling and *activin* signaling are not necessary for early left-right development. Coinjections of *follistatin* RNA and *activin* RNA confirm that follistatin is capable of inhibiting the effects of activin RNA injection on left-right development (Table 1A). Importantly, follistatin does not interfere with *Vg1* signaling (Schulte-Merker et al., 1994; Kessler and Melton, 1995). Coinjection of *follistatin* RNA with *BVg1* RNA does not block the ability of *BVg1* to invert the left-right axis (Table 1B), confirming that endogenous *Vg1* signaling is not inhibited by *follistatin* RNA injections. These results eliminate endogenous activin and BMPs as candidates for regulators of early left-right development.

Table 1. Coinjections of Signaling Antagonists

	Cardiac Reversal Rate (N)	
RNA-Injected	Into Cell L3	Into Cell R3
Experiment A		
Noggin	15% (20)	0% (18)
Follistatin	0% (53)	2% (48)
Activin	2% (65)	46% (61)
Coinjection of follistatin and activin	0% (74)	3% (75)
Experiment B		
BVg1	7% (15)	80% (20)
Coinjection of follistatin and BVg1	0% (6)	83% (12)
Experiment C		
BMP4	44% (63)	6% (54)
BVg1	0% (36)	83% (34)
Coinjection of BMP4 and BVg1	23% (47)	33% (46)

L3 or R3 cells were injected with the indicated combinations of RNAs. Percent of embryos with reversed cardiac orientation and number of embryos (N) are indicated. Uninjected controls had cardiac reversal rates from 2%–3%. L1 or R1 injection of noggin does not alter left-right development (Hyatt et al., 1996).

To further test the role of Vg1 in left-right development, an antagonist of Vg1 was sought. BMPs have been implicated in ventral mesoderm formation by antagonizing the dorsal organizing center (Dale et al., 1992; Sasai et al., 1995). Coinjection of *BMP4* and *BVg1* indicated that *BMP4* was capable of antagonizing the effects of *BVg1* on left-right development (Table 1C). Injection of *BMP2* or *BMP4* RNAs into the left side resulted in randomization of cardiac orientation (Figure 2D), as would be expected for treatments that antagonize the proposed *Vg1* signaling on the left side. Injection of *BMP2* or *BMP4* RNA into the right side (cell R2 or R3) had no significant effect on left-right development, further confirming that endogenous *BMP* signaling on the left is not involved in left-right development. These results suggest that expression of *BMPs* alters left-right development by interfering with endogenous *Vg1* signaling on the left side.

Compared to other treatments that randomize cardiac left-right orientation, *Xnr-1* expression patterns in *BMP*-injected embryos were altered in a novel way (Figure 4). In embryos in which cardiac orientation was randomized by *BMP2* RNA (Figure 2D), *Xnr-1* was expressed in a posterior domain on the left side but not in an anterior domain near the cardiac primordia (Figure 4A). There was no ectopic expression of *Xnr-1* on the right side. From these experiments, the normal *Xnr-1* expression pattern (Hyatt et al., 1996; Lowe et al., 1996; Lustig et al., 1996; Lohr et al., 1997) appears to be composed of two distinct but contiguous domains in the embryo. The anterior domain consists of a dorsal-ventral stripe just

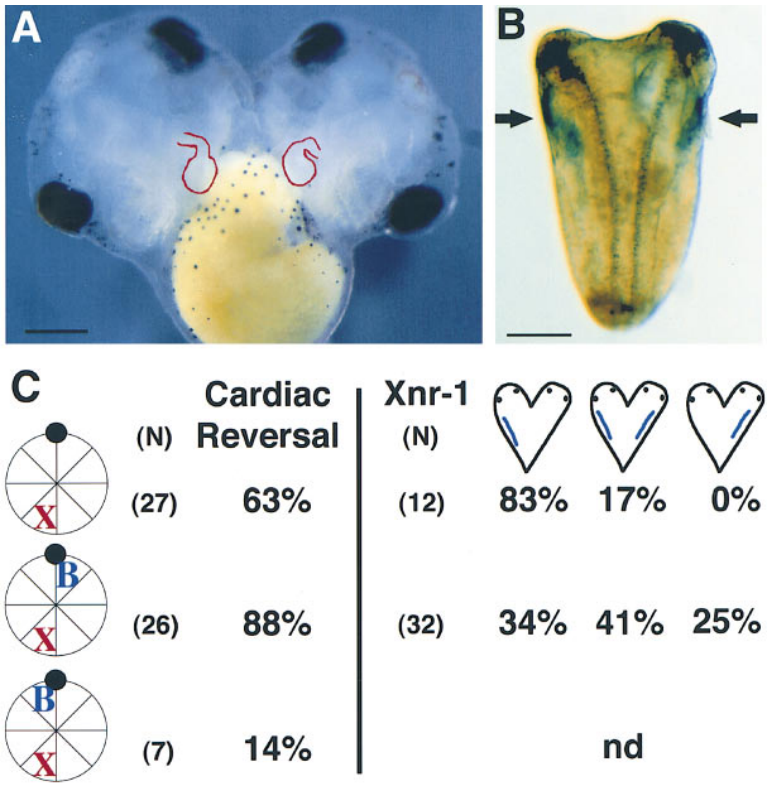

Figure 5. Lineage-Targeted Vg1 Expression Directs the Left-Right Axis in Conjoined Twins

(A) Ventral view of a *Xenopus* conjoined twin, produced by *Xwnt-8* RNA injection into L4 cell and *BVg1* RNA injection into the R1 cell. The left-side twin (on the right side of the panel) had a normally oriented heart. The right-side twin had a reversed heart, a mirror image of the left twin. Anterior is at top.

(B) Dorsal view of a *Xenopus* conjoined twin, showing in situ RNA analysis of *Xnr-1* expression pattern. *Xwnt-8* RNA was injected into the L4 cell and *BVg1* RNA injected into the R1 cell. *Xnr-1* was expressed predominantly in the left lateral plate of the left-side twin (arrow) and in the right lateral plate mesoderm of the right-side twin (arrow).

(C) Results of targeted *BVg1* expression in conjoined twins indicating cardiac reversal rates in the right twin and *Xnr-1* expression patterns. Conjoined twins were produced by *Xwnt-8* RNA injection into the L4 cell alone (first row) or in combination with *BVg1* RNA injection into the R1 cell (second row) or L1 cell (third row). Embryo drawings with blue lines represent categories of *Xnr-1* expression. N, number of embryos scored. In conjoined twins, heart orientation is randomized in the right-side twin (first row). *BVg1* injection into R1 inverted the left-right axis in the right twin and induced *Xnr-1* expression on the right side (second row). Injection of *BVg1* RNA into L1 rescued left-right axis formation to normal in the right twin (third row).

posterior to the pharyngeal arches (Figure 4B). In the absence of the anterior domain of *Xnr-1* expression on either side, cardiac orientation is randomized. The posterior domain is an anterior-posterior stripe just lateral to the somites along the posterior half of the embryo (Figure 4A). Injection of *BMP* into L2 results in a lower frequency of gut reversals than heart reversals (Figure 2D). These results suggest that the anterior expression domain of *Xnr-1* is necessary for normal left-right signaling to cardiac precursor cells and that the posterior domain regulates gut orientation.

Inversion and Rescue of the Left-Right Axis in Conjoined Twins

Spemann and Falkenburg (1919) demonstrated that conjoined amphibian twins could be created by a longitudinal ligature at blastula stages, indicating that the partially separated half-embryos could regulatively generate more complete embryonic axes. Strikingly, the left twins displayed normal left-right orientation and right twins displayed what would now be termed randomized left-right orientation. Similarly, human twins joined at the chest and/or abdomen (Levin et al., 1996), conjoined chick twins (Levin et al., 1996, 1997), and *Xenopus* twins created by injection of a variety of signaling molecules into the ventral side (Hyatt et al., 1996; Nascone and Mercola, 1997) display the same patterns; the left twin is normal and the right twin is randomized. Here, the randomized left-right orientation in the right twin serves as a model system in which to test the role of Vg1 in left-right axis formation. If Vg1 is capable of establishing a new left-right axis, placement of Vg1 on the left side of the right twin should rescue the randomized left-right axis to normal, and placement of Vg1 on the right side of the randomized twin should invert the left-right orientation.

Conjoined twins were formed by *Xwnt-8* RNA injection in the left ventral cell (L4) in 16-cell embryos. This creates a secondary embryonic axis on the left-side of primary axis, as assessed by lineage labeling (data not shown). The primary axis on the right side has normal (DAI 5) anterior-posterior and dorsal-ventral development (Figure 5A) and has randomized cardiac left-right orientation (Figure 5C, first row). In these conjoined twins (Nascone and Mercola, 1997), the left twins displayed predominate expression of *Xnr-1* in the left lateral plate mesoderm and correspondingly normal left-right orientation (Figure 5C). The majority of the right twins displayed no *Xnr-1* expression in lateral plate mesoderm and, correspondingly, had randomized cardiac orientation (Figure 5C, first row).

In order to test whether left-right axis formation in a right-sided twin, which is otherwise randomized, can be completely inverted by expression of Vg1 on its right side, conjoined twins were produced by *Xwnt-8* RNA injection into L4. Injection of *BVg1* RNA into R1 places Vg1 expression on the right side of the primary axis (which gives rise to the right-sided twin). Strikingly, left-right cardiac orientation and *Xnr-1* expression were inverted in the right twin (Figure 5C, second row), suggesting that placement of Vg1 on the right side of the otherwise randomized right twin completely inverts its left-right axis.

In order to test whether lineage-specific expression

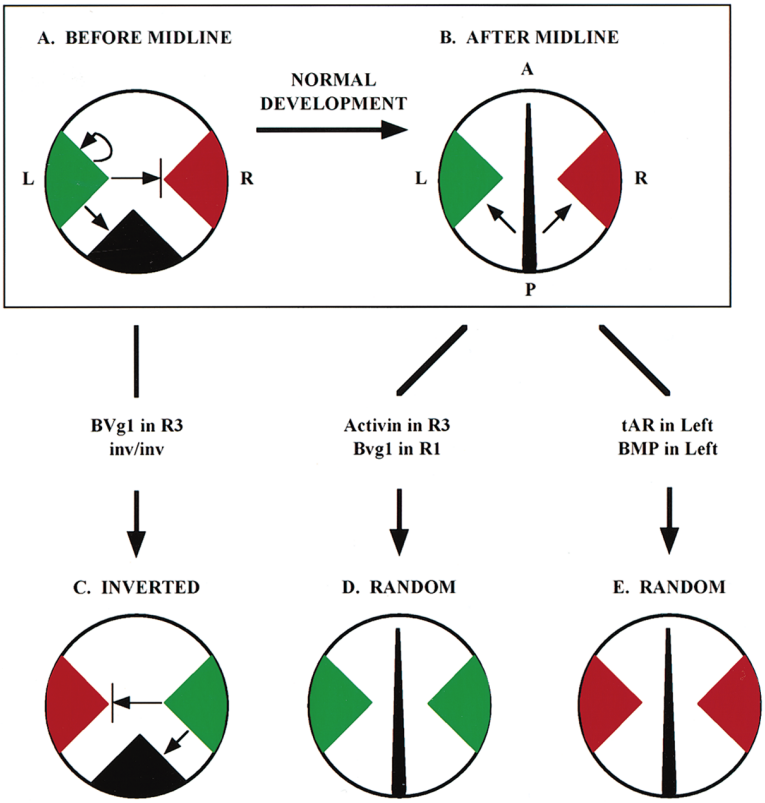

Figure 6. Model of the L-R Coordinator and a Role for Vg1

Green is left identity, red is right identity, and black is Spemann organizer and midline cells. See text for details.

(A) Normal left-right axis formation before midline development.

(B) Normal left-right axis formation after midline development separates the two sides.

(C) Inversion of the left-right axis.

(D) Randomization of the left-right axis, with two left-side (green) identities.

(E) Randomization of the left-right axis, with two right-side (red) identities.

of Vg1 can rescue normal left-right axis formation in an otherwise randomized embryo, conjoined twins were produced by *Xwnt-8* RNA injection (L4), and BVg1 RNA was injected into the L1 cell. This targets Vg1 expression to the immediate left side of the primary axis (which gives rise to the right-sided twin). Left-right cardiac orientation was rescued to normal orientation in these twins (Figure 5C, third row). These results suggests that the randomized state of right-sided twins is due to an absence of left-right signaling, which can be manipulated by targeted expression of the Vg1 signaling pathway to either rescue or completely invert the left-right axis, depending on which cell lineage is targeted in 16-cell embryos.

Discussion

A single alteration in Vg1 expression can invert the left-right axis, giving the left side of an embryo a right-side identity and the right side a left-side identity. In addition, randomization of left-right orientation in conjoined twins can be overridden by lineage-specific Vg1 expression. Remarkably, expression of Vg1 on the left side of an otherwise randomized embryo rescues left-right cardiac orientation to normal. Vg1 expression on the right side of an otherwise randomized embryo inverts cardiac left-right orientation. These results demonstrate that targeted Vg1 expression is sufficient to direct the orientation of left-right development.

Vg1 and the Left-Right Coordinator Model

Based on the ability to regulate the left-right axis by lineage-targeted *BVg1* expression in embryos and conjoined twins, we propose the following working model for left-right axis formation (Figure 6). A left-right (L-R) coordinator is located on the left side of the early embryo, in cells derived from the L3 cell in the 16-cell embryo (Figure 6A, green zone). This L-R coordinator is extrinsic to and orthogonal to the Spemann organizer, which establishes the anterior-posterior and dorsal-ventral axes. In normal development, activation of the L-R coordinator initiates left-side identity on the left side (green). The L-R coordinator also initiates right-side identity on the right side (red zone) by antagonistic signals from the left side (arrow with bar). In the sense that the L-R coordinator is capable of inducing left-right orientation throughout the embryo, it serves as an "organizer" of left-right development. However, in the absence of L-R coordinator activity, left-right asymmetries are still generated (i.e., the cardiac tube loops) but left-right organ orientation is randomized. Thus, the L-R coordinator model does not explain the biomechanical generation of morphological asymmetry (i.e., tube looping) but has the utility of explaining the evolutionarily conserved *coordination* of left-right asymmetries with the other embryonic axes.

The mechanism by which the L-R coordinator is placed on the left side is unknown, but the observation that a transient array of microtubules during the first cell cycle are essential for normal left-right development suggests that the L-R coordinator is initiated at the same time as dorsal ventral axis formation (Yost, 1991, 1995). It is likely that the L-R coordinator transmits left-right axis information to the Spemann organizer (as indicated by arrow from green zone to black zone in Figure 6A) and that the Spemann organizer subsequently assumes the role of directing left-right development, including

sending signals back out to tissues developing in lateral positions (as indicated by arrows from midline in Figure 6B). Several genes, including *Shh*, are asymmetrically expressed in the node in chick (analogous to the Spemann organizer in amphibians), and alteration of these expression patterns perturbs subsequent left-right development in lateral tissues (Levin et al., 1995, 1997). Two recent observations in chick concur with the suggestion that left-right asymmetries in the Spemann organizer (node in chick) are established by signals from lateral cells, i.e., from the L-R coordinator. First, a node with normal left-right gene expression can be regenerated in an embryo from which the endogenous node was deleted (Psychoyos and Stern, 1996), suggesting that left-right asymmetries in the lateral cells can correctly pattern a regenerated node. Second, the asymmetric expression pattern of *Shh* in a node that was surgically inverted early in embryogenesis is oriented with respect to the lateral tissues, i.e., the L-R coordinator, not with respect to the initial node orientation (Pagán-Westphal and Tabin, 1998 [this issue of *Cell*]).

The proposed L-R coordinator model is consistent with the molecular and morphological results reported here. Expression of Vg1 on the opposite side (cell R3) of the L-R coordinator inverts the left-right axis (Figure 6C), turning the geometric right side into left identity (green), which then converts the geometric left into right (red), giving a mirror image of the normal embryo. The inversion of the left-right axis is reflected both morphologically and by the inversion of *Xnr-1* expression patterns. We propose that expression of Vg1 in R3 initiates the formation of an L-R coordinator on the right side, which can suppress the formation of an L-R coordinator on the left side. The L-R coordinator is physically distinct from the Spemann organizer, as exemplified by the observation that the strongest effect of Vg1 expression is in R3, which is a greater distance from the Organizer than R2 or R1.

No other ectopic expressions of signaling molecules in *Xenopus* or chick embryos are known to invert the left-right axis; at best they result in randomization of left-right orientation. In the model, randomization results from conversion of identity on only one side of the midline. For example, expression of activin on the right side converts right into left (red into green, Figure 6D) without altering the identity on the other side of the midline. Both sides acquire left identity (two green sides) and express Xnr-1; the resulting morphological outcome is determined stochastically (i.e., organ orientation is random). Similarly, expression of Vg1 antagonists (*tAR*, *BMP2*, and *BMP4*) on the left side reduces activity downstream of the L-R coordinator on the left side, resulting in embryos with two right sides (two red sides), again yielding random orientation of internal organs (Figure 6).

The proposed model of an L-R coordinator is strengthened by the results in conjoined twins. The absence of *Xnr-1* expression on either side of the right twin's midline (83% of cases, Figure 5C, row 1) (Nascone and Mercola, 1997) suggests that the randomization of cardiac orientation in the right-sided twin is due to the absence of an L-R coordinator near the right twin's Organizer, such that both sides develop right-side identity (red in Figure 6E). The cardiac reversal rate of 63% in right-sided twins is predicted by the *Xnr-1* expression patterns. If absence of Xnr-1 (83% of right-sided twins) results in randomization and right-sided *Xnr-1* (17% of right-sided twins) yields full inversion, the predicted reversal rate would be 58.5% (17% plus half of 83%). The absence of L-R coordinator in the right twin could be due to separation from the endogenous L-R coordinator by the intervening left twin (which has a normal left-right axis and a normal Xnr-1 expression pattern) or to inhibition by the midline of the left twin.

Placement of L-R coordinator activity (by *BVg1* injection) on the right twin's right side induces *Xnr-1* on the right side (66% of the cases, Figure 5C, row 2) and inverts cardiac orientation. In 25% of the cases, expression of Vg1 on the right side of the right twin not only induces *Xnr-1* expression on the right side, but apparently is capable of preventing *Xnr-1* expression on the left side of the left twin (Figure 5C, row 2). This is not surprising, in light of the ability of injected *BVg1* to invert the left-right axis throughout the normal (non-twin) embryos. Placement of new L-R coordinator activity on the right twin's left side rescues left-right axis formation (Figure 5C, row 3). There are no other known examples of rescuing an embryo to normal left-right orientation.

Controlling Left-Right Development by Lineage-Specific Vg1 Expression and a Model for the *INV* Gene

The inverted phenotype caused by *BVg1* injection in the R3 cell is analogous to the phenotype in *inv/inv* mice (Yokoyama et al., 1993) but has not previously been obtained by experimental manipulation of a molecular signaling pathway. The molecular nature of the *inv* lesion has not been identified, but complex models have been invoked to explain the molecular mechanism that inverts the left-right axis in *inv/inv* mice, including loss of a pathway that uncovers a default pathway (Yokoyama et al., 1993) and a chromosome inversion that alters chromatid segregation (Klar, 1994). Our results suggest that the molecular mechanism that leads to the *inv/inv* phenotype could be as simple as the activation of the *Vg1* signaling pathway in additional and inappropriate cell lineages on the right side of the embryo, without alteration of the normal Vg1 expression on the left side. Expression of Vg1 in the R3 lineage is sufficient to activate L-R coordinator activity on the right side of the embryo, including activation of *Xnr-1* expression in the right lateral plate, and to cause right-side signaling on the left side of the embryo, leading to absence of *Xnr-1* expression in the left-lateral plate. A molecular consequence of the inv mutation simply might be to broaden the expression zone of Vg1, leading to expression of Vg1 in aberrant cell lineages.

Members of the TGFβ Family Have Distinct Roles in Left-Right Lineages Established in 16-Cell Embryos; Only Vg1 Initiates the L-R Coordinator

The effects on left-right development of injected signaling molecules are cell-lineage dependent (Figure 2). In L1 through L3, expression of *BVg1* or *activin* has no

effect, whereas expression of *BMP2*, *BMP4*, or a dominant negative receptor (*tAR*) randomizes left-right orientation, consistent with these molecules antagonizing Vg1 on the left side. In R1, expression of Vg1 randomizes left-right orientation, whereas expression of activin does not. In R3, expression of Vg1 inverts left-right orientation, whereas expression of activin randomizes left-right orientation. It is striking that adjacent sister cells respond distinctly to expression of identical signals. These results emphasize the importance of targeting specific cell lineages in expression studies and suggest that distinct lineages are established along the left-right axis in 16-cell embryos.

Clearly members of the TGFβ superfamily, including Vg1, activin, nodal, lefty, and BMPs, have roles at various stages in left-right development (Levin, 1997). However, only Vg1 protein expression on the right side is capable of inverting the left-right axis. *Nodal* and *lefty* are expressed late in left-right development, making it unlikely that they are involved in the first steps in left-right axis formation. Furthermore, expression of *Xenopus* nodal (*Xnr-1*) after the mid-blastula stage on the right side can randomize but not invert left-right orientation (Sampath et al., 1997). Manipulations of the activin pathway by *activin* expression in R3 (Figure 2B), perturbation of activin receptor activity on the left side (Figure 2C), or *activin IIB receptor* gene knockout in mice (Oh and Li, 1997) only randomize left-right orientation, suggesting that *activin* may play a downstream role in left-right development. Follistatin blocks activin, but not *BVg1*, and does not alter left-right development (Table 1), indicating that *activin* signaling on either side of the embryo is not necessary for early left-right axis formation. Injection of *noggin*, which binds and antagonizes BMPs (Zimmerman et al., 1996), does not alter left-right development, indicating that endogenous BMPs are not necessary for left-right axis formation. Instead, we show that *BMP4* acts as an antagonist of Vg1 (Table 1C), suggesting that injection of *BMP* on the left randomizes left-right development (Figure 2D) by antagonizing endogenous Vg1 on the left. Thus, among the TGFβ superfamily members known to be expressed in early embryos, endogenous Vg1 is most likely to initiate the L-R coordinator. Inhibition of Vg1 alters left-right development, and expression of Vg1 in the R3 cell results in an unprecedented inversion of the left-right axis, indicating that Vg1 is an early determinant in left-right axis formation.

Experimental Procedures

Nomenclature and Microinjection of *Xenopus* Embryos

A nomenclature for the vegetal cells of a 16-cell stage *Xenopus* embryo (Figures 1A–1B) was modified from Hirose and Jacobson (1979), Nakamura and Kishiyama (1971), and Moody (1987). The left and right sides are identified as L and R, and cells numbered 1 to 4 from dorsal to ventral. The future dorsal midline in *Xenopus laevis* embryos was stained with a Nile blue dot during the first cell cycle, as previously described (Danos and Yost, 1995; Hyatt et al., 1996). RNA injections (in 4.7 nl) were: 200–400 pg *BVg1*, 2 ng Vg1, 400 pg *BVg1–2cx*, 200–400 pg *AVg*, 0.5–1 pg *activin*, 1.5–2 ng *tAR*, 1 ng *follistatin*, 100 pg *noggin*, 2 ng *BMP2*, 400–500 pg *BMP4*. Higher doses of some of these RNAs had adverse effects on development. Conjoined twins were created by injection of *Xwnt-8* RNA (25–100 pg) mixed with conjugated rhodamine-dextran lineage label (5 mg/ml). *pXEX-BVg1* was made by PCR amplification of *BVg1* from *pSP64TBVg1* (Thomsen and Melton, 1993) with a 5′ primer, 5′-GGGGTACCGAAAACCCACATCGAGAC-3′ (KpnI site underlined), and a 3′ primer, 5′-CCATCGATTGGCACATATGGTCACC-3′ (ClaI site underlined), and insertion into KpnI and ClaI sites in *pXeX* (Johnson and Krieg, 1994). *pXEX-BVg1* or control *pXEX* (50–200 pg) were injected into embryos.

Embryo and Data Analysis

In gastrula, the dorsal lip formed at the Nile blue dot, indicating that injections did not shift the location of the dorsal midline. Post–stage 43 embryos were assessed for heart and viscera orientation (Yost, 1992); other aspects of development, including dorsoanterior development (Danos and Yost, 1995; Hyatt et al., 1996), were normal. *Xnr-1* whole-mount in situ hybridization of stage 24–26 embryos was performed as described (Hyatt et al., 1996). For analysis in conjoined twins, only embryos with a primary axis on the right side and a secondary axis (identified by lineage label) with a DAI of 4 or above (Danos and Yost, 1995) were scored. Statistical significance was set at $P < 0.05$ in standard chi-squared tests of independence or goodness of fit and/or z-test of independent proportions as in Lohr et al. (1997).

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